

Sphingosine-1-phosphate receptors control B-cell migration through signaling components associated with primary immunodeficiencies, chronic lymphocytic leukemia, and multiple sclerosis

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Background: Five different G protein–coupled sphingosine-1-phosphate (S1P) receptors (S1P1–S1P5) regulate a variety of physiologic and pathophysiologic processes, including lymphocyte circulation, multiple sclerosis (MS), and cancer. Although B-lymphocyte circulation plays an important role in these processes and is essential for normal immune responses, little is known about S1P receptors in human B cells. **Objective:** To explore their function and signaling, we studied B-cell lines and primary B cells from control subjects, patients

with leukemia, patients with S1P receptor inhibitor–treated MS, and patients with primary immunodeficiencies.

Methods: S1P receptor expression was analyzed by using multicolor immunofluorescence microscopy and quantitative PCR. Transwell assays were used to study cell migration. S1P receptor internalization was visualized by means of time-lapse imaging with fluorescent S1P receptor fusion proteins expressed by using lentiviral gene transfer. B-lymphocyte subsets were characterized by means of flow cytometry and immunofluorescence microscopy.

Results: Showing that different B-cell populations express different combinations of S1P receptors, we found that S1P1 promotes migration, whereas S1P4 modulates and S1P2 inhibits S1P1 signals. Expression of CD69 in activated B lymphocytes and B cells from patients with chronic lymphocytic leukemia inhibited S1P-induced migration. Studying B-cell lines, normal B lymphocytes, and B cells from patients with primary immunodeficiencies, we identified Bruton tyrosine kinase, β -arrestin 2, LPS-responsive beige-like anchor protein, dedicator of cytokinesis 8, and Wiskott-Aldrich syndrome protein as critical signaling components downstream of S1P1. **Conclusion:** Thus S1P receptor signaling regulates human B-cell circulation and might be a factor contributing to the pathology of MS, chronic lymphocytic leukemia, and primary immunodeficiencies. (*J Allergy Clin Immunol* 2014;■■■:■■■-■■■.)

Key words: Sphingosine-1-phosphate, B cells, migration, autoimmunity, circulation, fingolimod, FTY720, primary immunodeficiencies

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The phospholipid sphingosine-1-phosphate (S1P) binds to 5 different G protein–coupled receptors (ie, S1P1–S1P5) and acts as a central signaling molecule in cellular responses, including lymphocyte migration.¹

In mice S1P binding to S1P1 triggers the exit of B cells from secondary lymphoid tissues,² the shuttle of marginal zone (MZ) B cells between follicles and the MZ in the spleen,³ and the egress of immature B cells from and migration of plasma cells (PCs) to the bone marrow.^{4–6} S1P3 induces migration of immature and MZ B cells,^{3,7,8} whereas S1P2 antagonizes chemokine receptor signaling of mouse lymphocytes, thus keeping B cells inside germinal centers (GCs).⁹ S1P4 seems to modulate murine T-cell migration but has little effect on mouse B-cell migration.¹⁰

Abbreviations used

ALL:	Acute lymphoblastic leukemia
B-CLL:	B-cell chronic lymphocytic leukemia
BCR:	B-cell receptor
BFP:	Blue fluorescent protein
BTK:	Bruton tyrosine kinase
cALL:	Common lymphocytic leukemia
DMSO:	Dimethyl sulfoxide
DOCK8:	Dedicator of cytokinesis 8
FITC:	Fluorescein isothiocyanate
GC:	Germinal center
GEF:	Guanine nucleotide exchange factor
GFP:	Green fluorescent protein
LRBA:	LPS-responsive beige-like anchor protein
MACS:	Magnetic cell sorting
MS:	Multiple sclerosis
MZ:	Marginal zone
qPCR:	Quantitative PCR
PC:	Plasma cell
PE:	Phycoerythrin
RFP:	Red fluorescent protein
RhoGAP:	RhoGTPase activating protein
ROCK:	Rho-associated protein kinase
S1P:	Sphingosine-1-phosphate
TIRFM:	Total internal reflection fluorescence microscopy
TLR:	Toll-like receptor
WAS:	Wiskott-Aldrich syndrome
WASp:	Wiskott-Aldrich syndrome protein
WIP:	Wiskott-Aldrich syndrome protein-interacting protein

The phosphorylated form of the S1P analog FTY720 (fingolimod) binds to all S1P receptors, except S1P2.¹¹ It blocks lymphocyte egress and circulation because S1P-metabolizing enzymes cannot degrade FTY720. This masks the natural S1P gradient,^{12,13} and currently, FTY720 is used successfully in the treatment of multiple sclerosis (MS), a T and B cell-driven autoimmune disease of the central nervous system.¹⁴

Several genetic defects, including Wiskott-Aldrich syndrome (WAS)¹⁵ and Wiskott-Aldrich syndrome protein-interacting protein (WIP) deficiency,¹⁶ impair the organization of the cytoskeleton and result in primary immunodeficiencies. Because Wiskott-Aldrich syndrome protein (WASp) regulates actin polymerization and filament branching, mutations in *WAS* lead to defects in lymphocyte migration,¹⁷ break B-cell tolerance, and result in autoimmunity.¹⁸ Upstream of WASp, dedicator of cytokinesis 8 (DOCK8) acts as a guanine nucleotide exchange factor (GEF) for CDC42.¹⁹ In mice DOCK8-defective cells cannot form immunologic synapses, resulting in impaired T-cell survival and function.²⁰ In human subjects mutations in *DOCK8* cause autosomal recessive hyper-IgE syndrome,²¹ impair lymphocyte activation, and might correlate with reduced numbers of circulating lymphocytes.²² It is not known whether DOCK8-associated lymphopenia is caused by defects in cell migration. Other immunodeficiencies affecting proteins involved in vesicle transport, such as the LPS-responsive beige-like anchor protein (LRBA),^{3,24} or in the RhoGTPase activating proteins (RhoGAPs) regulating cell motility (eg, ARHGAP4)^{25,26} might impair S1P receptor signaling. Mutations in *LRBA* cause severe, early-onset humoral immunodeficiency and autoimmunity.²³ ARHGAP4 is localized at the leading edge of motile cells and inhibits cell migration and axon outgrowth.²⁵ ARHGAP4 deficiency results in primary

immunodeficiency that is characterized by low numbers of circulating T cells and almost complete absence of CD8⁺ T cells.²⁶

Studying primary B lymphocytes and EBV-immortalized B cells, we report that differentially expressed S1P receptors regulate human B-cell migration. Analyzing cells of different primary immunodeficiencies, we show that the signaling cascade induced by S1P1 ranges from β -arrestin 2 through LRBA and DOCK8 to WASp. S1P4 was found to modulate S1P1 activity, whereas expression of S1P2, as well as activation of resting B cells or malignant transformation, strongly inhibited S1P1-dependent migration.

Thus S1P receptors regulate the circulation of normal and autoimmune B cells in human subjects and play an important role in the clinical manifestations of primary immunodeficiencies and B-cell malignancies.

METHODS

The [Methods](http://www.jacionline.org) section in this article's Online Repository at www.jacionline.org contains detailed information on patients, materials, and methods.

RESULTS**S1P receptor expression in primary human B-cell subsets**

Because the expression pattern of S1P receptors in human B cells was unknown, we analyzed S1P receptor expression and S1P-dependent migration in B cells from lymphoid tissue and blood. In tonsils S1P1 was detected in IgD⁺ mantle zone B cells but not in CD38⁺ extrafollicular PCs or GC B cells ([Fig 1, A](#), and see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org). In the spleen CD19⁺IgM^{lo}IgD^{high} mantle zone and IgM^{high}-IgD^{lo/-} MZ B cells expressed S1P1 ([Fig 1, B](#), and see [Fig E1, B](#)). Because immunostaining of S1P2 to S1P5 was either unspecific or less than detection limits, we analyzed expression of all S1P receptors using quantitative PCR (qPCR) in purified tonsillar and blood B-cell subsets. Naive and memory tonsillar B cells expressed comparable levels of S1P1 and S1P4 but 50 to 100 times fewer S1P2 transcripts. GC B cells mainly expressed S1P2 and S1P4 but 10 to 100 times less S1P1 than naive/follicular B cells residing in the IgD⁺ mantle zone, whereas PCs expressed S1P1, S1P2, and S1P4 at similar levels ([Fig 1, C](#)). Blood B-cell subsets expressed high levels of S1P1 and S1P4, although in transitional B cells the expression was 10 to 20 times lower. Transitional, naive, and memory B cells also expressed S1P2, but the signals were 50 to 100 times weaker, as for the other S1P receptors ([Fig 1, D](#)). In contrast to murine immature and MZ B cells, which express functional S1P3,³ S1P3 transcripts were not detected in human B cells. S1P5-specific transcripts were less than detection levels.

The subset-specific expression pattern of S1P receptors reflected differences in the response to S1P. S1P1⁺, naive, and memory B cells from the tonsils, as well as naive, memory, transitional, and MZ B cells from the blood, migrated efficiently in Transwell assays ([Fig 1, E and F](#)), whereas S1P2⁺ GC B cells did not migrate and the spontaneous motility of S1P1⁺S1P2⁺S1P4⁺ PCs was inhibited ([Fig 1, E](#)).

We then analyzed the response of human B cells to S1P *in vivo* over a period of 60 days by determining the absolute numbers and percentages of B cells in patients with MS before and during treatment with the S1P analog FTY720. In contrast to the progressively decreasing numbers of transitional, naive,

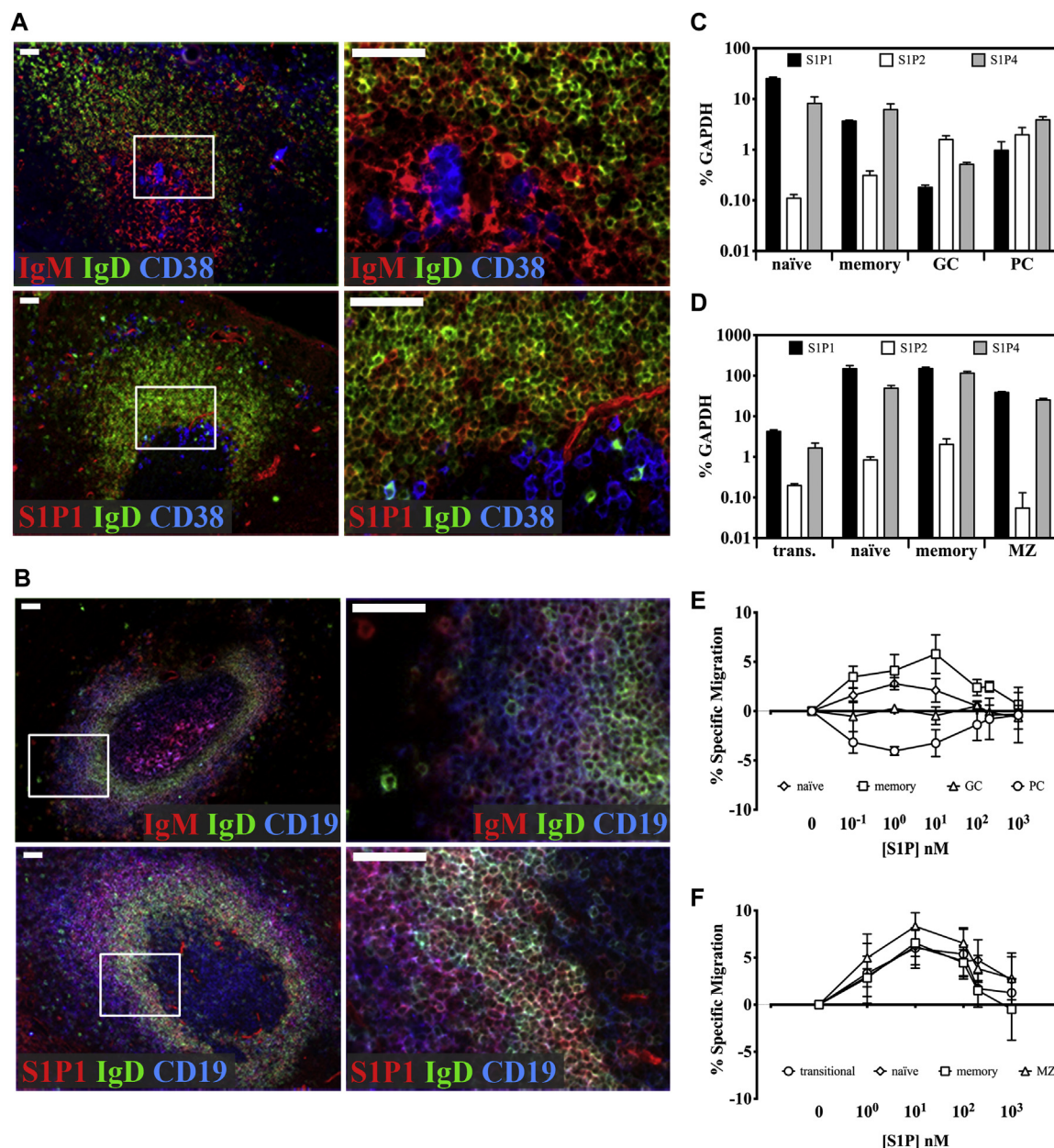


FIG 1. Distinct S1P receptor expression patterns allow migration of human B cells. **A** and **B**, Immunofluorescence microscopy of tonsillar (Fig 1, **A**) and spleen (Fig 1, **B**) cryosections. Scale bars = 50 μ m. **C** and **D**, S1P receptor mRNA expression in B-cell subsets from tonsillar (Fig 1, **C**) and blood (Fig 1, **D**). Values are means $\pm$ SDs of 2 technical replicates representative of 3 biological replicates. **E** and **F**, Migration of tonsillar (Fig 1, **E**) and blood (Fig 1, **F**) B-cell subsets. B-cell subsets were gated as shown in Fig E11. Primer sets are listed in Table E1. Values are presented as means $\pm$ SEMs of 4 (Fig 1, **E**) and 5 (Fig 1, **F**) different biological replicates. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

MZ, and switched memory B cells, the numbers of circulating PCs remained unchanged (Fig 2). Thus in human subjects S1P1 and S1P4 regulate the circulation of naïve and memory B cells, the egress of transitional B cells from the bone marrow, and the exit of MZ B cells from the spleen.

S1P receptor signaling in activated and leukemic B cells

Antigen binding and cellular activation inhibit S1P-dependent B-cell migration through direct interactions between CD69 and

S1P1.²⁷ Stimulation of human primary B cells *in vitro* with anti-IgM, CD40 ligand, and CpG resulted in downregulation of S1P1 mRNA levels (Fig 3, **A**), induced CD69 expression, and inhibited S1P-dependent B-cell migration (Fig 3, **B**).

Because transformed B cells resemble activated cells to some extent,²⁸ we studied migration and S1P receptor expression patterns in B cells from patients with leukemia. CLL, pre-B-acute lymphoblastic leukemia (ALL), and common lymphocytic leukemia (cALL) cells expressed S1P4 at similar levels to control B cells, whereas signals of S1P1 transcripts were 10 times lower (Fig 3, **C**), approximating levels found in transitional B cells.

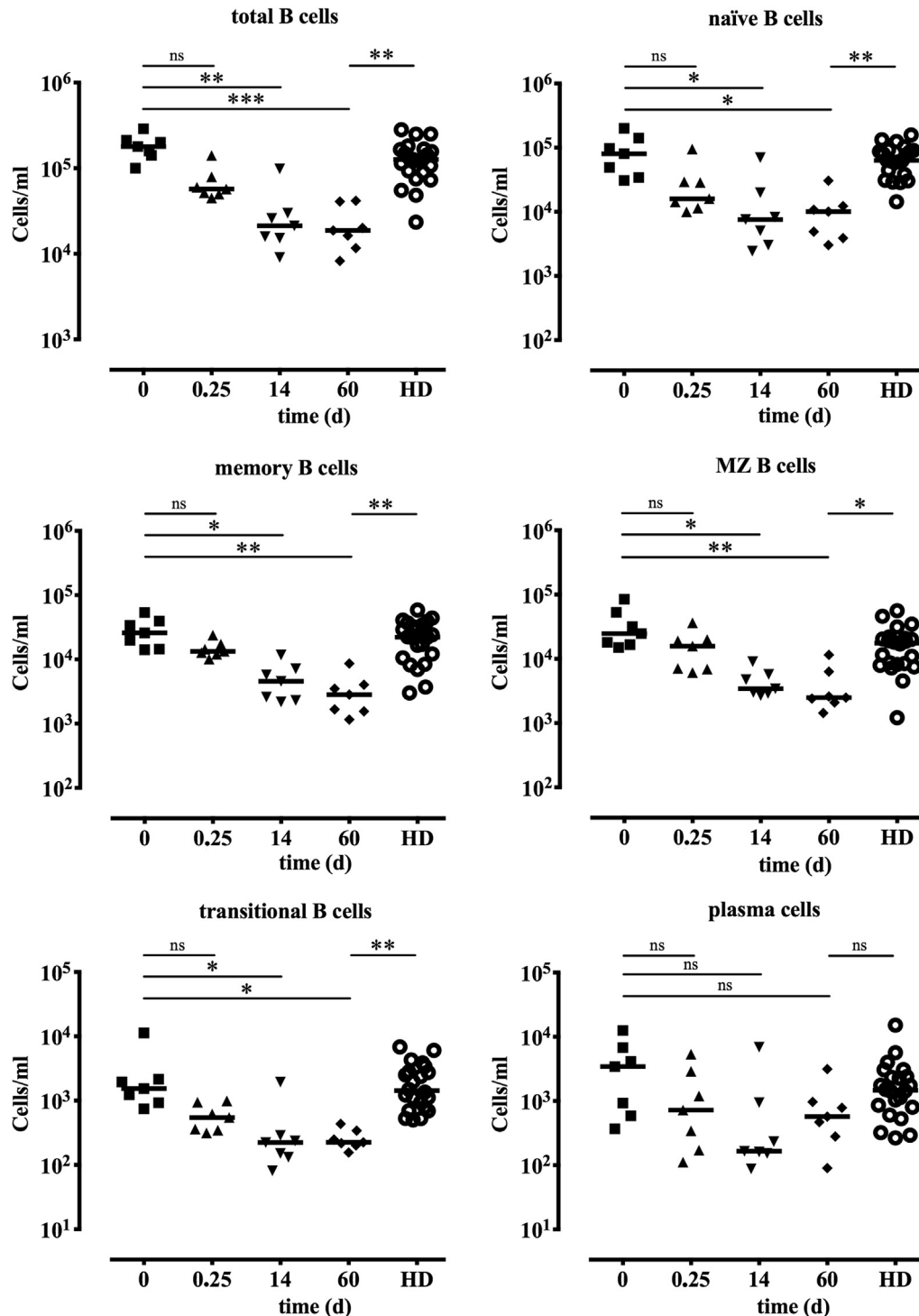


FIG 2. FTY720 blocks B-cell egress from bone marrow and secondary lymphoid organs. The decrease in absolute numbers of circulating total, transitional, naïve, switched memory, and MZ B cells and of PCs in healthy donors (HDs) and FTY720-treated patients with MS under fingolimod therapy is shown. Each symbol represents an individual patient or healthy donor. Small horizontal lines indicate the median. * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

However, none of the cells from patients with leukemia migrated as efficiently toward S1P as transitional B cells (Fig 3, D). Surprisingly, nonstimulated CD69⁺ B cells of healthy donors migrated toward S1P, although not as efficiently as their CD69[−] counterparts, whereas CD69[−] CLL cells migrated only poorly and

CD69⁺ CLL cells did not migrate (see Fig E2, A, in this article's Online Repository at www.jacionline.org). This indicates that CD69 expression alone is not sufficient to block S1P migration of B-cell chronic lymphocytic leukemia (B-CLL) cells. In patients with CLL, inhibition of chronic B-cell receptor (BCR)

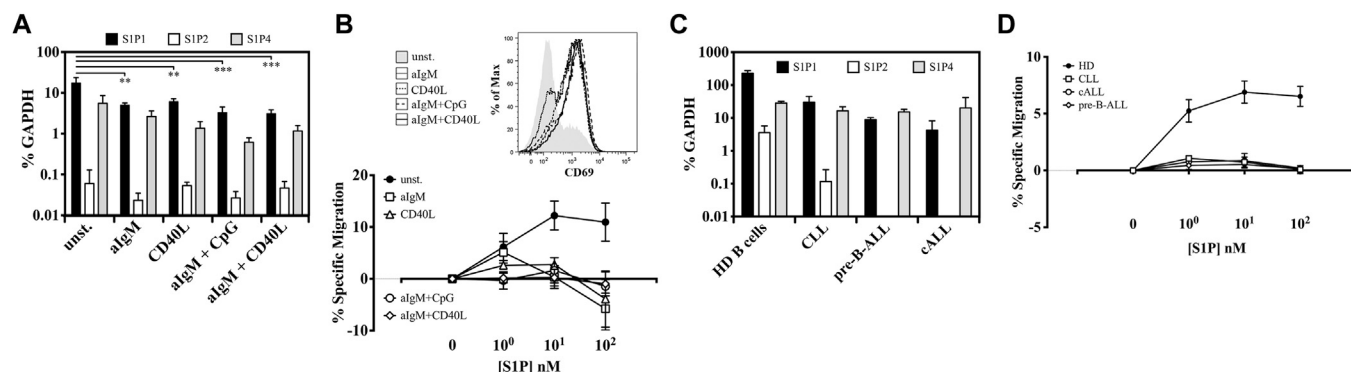


FIG 3. Activation and malignancy reduce S1P receptor expression and migration of B cells. **A**, S1P receptor mRNA expression in B cells 14 hours after activation with anti-IgM, CD40 ligand (CD40L), and CpG. *unst.*, Unstimulated. Values are means $\pm$ SDs of 3 different biological replicates. **B**, CD69 expression (top) and migration (bottom) of B cells activated as in Fig 3, A. Values are means $\pm$ SEMs of 3 different biological replicates. **C**, S1P receptor mRNA expression in CLL, pre-B-ALL, and cALL cells. Values are means $\pm$ SDs of 3 different biological (CLL) and 2 technical replicates (healthy donor [HD], pre-B-ALL, and cALL cells). **D**, Migration of CLL, pre-B-ALL, and cALL B cells. Values are means $\pm$ SEMs of 12 (healthy donor cells [HD]) and 5 (CLL cells) different and 1 (pre-B-ALL and cALL cells) biological replicates.

signaling by the Bruton tyrosine kinase (BTK)–specific inhibitor PCI-32765 leads to the massive exit of leukemic B cells from lymphoid tissues.²⁹ Therefore we speculated that BTK inhibition might restore the ability of CD69⁺ B cells to respond to S1P. As expected, PCI-32765 treatment of anti-BCR–activated B cells decreased CD69 expression of nonstimulated B cells and anti-BCR–activated B cells, but to our surprise, it blocked S1P-induced migration of CD69⁺ B cells (see Fig E2, B). PCI-32765 treatment of CLL cells for up to 40 hours neither decreased CD69 expression in all CLL samples nor restored S1P migration (see Fig E3 in this article’s Online Repository at www.jacionline.org). Thus BTK activity seems to be required for S1P-induced migration.

S1P2 and S1P4 regulate S1P1-induced migration

Analyzing S1P receptor expression in cell lines established from patients with B leukemia, lymphoma, or myeloma, we detected S1P1 expression in the pre-B-ALL line Nalm6, the EBV-transformed lymphoblast line Dakiki, and the myeloma line KMS-12 (see Fig E4, A, in this article’s Online Repository at www.jacionline.org). S1P2 was expressed in all diffuse large B-cell lymphomas, some Burkitt lymphoma lines, and Nalm6. S1P4 was expressed in all cell lines except for the Burkitt lymphoma line BJAB and the diffuse large B-cell lymphoma lines (see Fig E4, A). Because only S1P1⁺ Dakiki cells migrated *in vitro* (see Fig E4, B), we tested whether S1P-driven migration is S1P1 dependent and transduced S1P1⁺ Ramos cells with a lentiviral S1P1–enhanced green fluorescent protein (GFP) fusion protein expression vector. As in mouse B cells, S1P1 expression in human B-cell lines induced migration toward S1P (see Fig E4, C).

Because the Nalm6 and KMS-12 lines coexpressed S1P1 together with S1P2 or S1P4 but did not migrate like Dakiki cells, which expressed more S1P1 than S1P4, we tested whether the balance between S1P2 or S1P4 and S1P1 regulates migration to S1P. S1P1⁺ Dakiki cells transduced with S1P2-GFP did not migrate (see Fig E4, D), and similarly, BJAB cells cotransduced with S1P1–red fluorescent protein (RFP) and S1P2-GFP migrated less efficiently than S1P1-RFP⁺ cells (see Fig E4, E). To test

whether S1P binding to S1P2 is needed to block S1P1 activity, we performed migration assays and time-lapse microscopy of transduced cells using FTY720P as a chemoattractant. In contrast to S1P, FTY720P did not block S1P1-driven migration of S1P1⁺S1P2⁺ cells (see Fig E5 in this article’s Online Repository at www.jacionline.org), thus showing that activation of S1P2 is needed to inhibit S1P1. Because the activation of S1P1 with FTY720P did not induce the cointernalization of S1P2–blue fluorescent protein (BFP), both receptors do not seem to interact directly but rather to signal independently (see Video E1 in this article’s Online Repository at www.jacionline.org).

S1P4 was endogenously coexpressed with S1P1 in Nalm6, Dakiki, and KMS-12 cells. S1P4 overexpression alone did not induce migration toward S1P and did not interfere with S1P1 expression. However, S1P4 overexpression reduced S1P1-driven migration, although not to the same extent as S1P2 (see Fig E4, F, and Fig E6 in this article’s Online Repository at www.jacionline.org).

Because S1P1 is internalized, we studied the early steps and dynamics of S1P1, S1P2, and S1P4 endocytosis using total internal reflection fluorescence microscopy (TIRFM). Time-lapse imaging revealed that S1P rapidly induced receptor clustering, followed by endocytosis starting from 5 to 6 minutes after stimulation (see Videos E2 and E3 and Fig E7 in this article’s Online Repository at www.jacionline.org). S1P4-GFP clusters and vesicles appeared later than those formed by S1P1 and S1P2. Because the clusters contained 1 or more S1P receptor species (see Video E3), receptor internalization occurs independently for each receptor, nevertheless allowing cointernalization of different receptors into the same vesicles.

Thus S1P binding to S1P1, S1P2, and S1P4 initiates rapid clustering in vesicles that are internalized. S1P1-induced migration is blocked by coexpression and simultaneous activation of S1P2. In contrast to S1P2, S1P4 only modulates S1P1-induced migration.

β -Arrestin 2 increases S1P1-induced migration

Internalization of G protein–coupled receptors involves binding of β -arrestin 1 or β -arrestin 2 and results either in receptor desensitization or recruitment of additional signaling

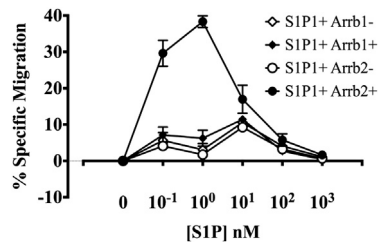


FIG 4. S1P-dependent migration is enhanced by β -arrestin 2. Migration of S1P1 and β -arrestin 1-transduced (*Arrb1*) or S1P1 and β -arrestin 2-transduced (*Arrb2*) Ramos cells. Values are means $\pm$ SEMs of 3 biological replicates.

molecules to the scaffold formed by arrestins.³⁰ Activation of S1P1, S1P2, or S1P4 in β -arrestin 2-expressing cells recruited β -arrestin 2 to S1P receptor-containing vesicles located close to the plasma membrane, followed by rapid internalization (see Video E4 in this article's Online Repository at www.jacionline.org). Strikingly, coexpression with β -arrestin 2 not only enhanced but also shifted the sensitivity of S1P1-mediated migration from 10 nmol/L to 0.1 to 1 nmol/L S1P (Fig 4). Like β -arrestin 2, β -arrestin 1 was recruited to S1P receptor-containing vesicles (see Video E5 in this article's Online Repository at www.jacionline.org), but in contrast to β -arrestin 2, it neither enhanced migration nor increased the sensitivity for S1P (Fig 4).

The RhoGAP ARHGAP4 localizes to the leading edge of migrating NIH3T3 fibroblasts and at axon growth cones, where it acts as a potent inhibitor of cell motility.²⁵ Analyzing the S1P-dependent migration of EBV-immortalized B cells from ARHGAP4-deficient patients, we did not observe any differences compared with EBV cells from healthy donors (see Fig E8, A, in this article's Online Repository at www.jacionline.org). S1P2 activation inhibits RAC activity through the antagonizing function of RhoGTPase, which is regulated by Rho-GEFs and RhoGAPs.⁹ Because ARHGAP4 was found to inhibit cell motility and migration, we analyzed whether ARHGAP4 would be a downstream signaling component of S1P2. Comparing the migration of S1P2-BFP-expressing ARHGAP4-deficient and wild-type EBV cells, we did not detect significant differences in the S1P2-induced inhibition between normal and ARHGAP4-deficient cells *in vitro* (see Fig E8, A).

S1P migration of S1P2⁺ glioblastoma cells is inhibited through the Rho-associated protein kinase (ROCK) pathway.³¹ Although inhibition of ROCK inhibited the migration of S1P1⁺ Ramos cells, it did not change the S1P2-dependent blockade of S1P1 activity (see Fig E8, B).

Thus ARHGAP4 activity is dispensable for S1P1 or S1P2 signaling in EBV-immortalized B cells. Similarly, ROCK signaling seems not to be important for S1P2-mediated inhibition of migration in B cells.

DOCK8, WASp, and LRBA are components of the S1P1-signaling cascade

Several primary immunodeficiencies are caused by mutations affecting the function of cytoskeleton-interacting protein. In addition to primary B cells, we used EBV lines from immunodeficient patients to study the role of these components in S1P receptor signaling because EBV immortalization of primary B cells enhances S1P-induced migration but does not alter the

overall migration pattern (see Fig E9 in this article's Online Repository at www.jacionline.org).

The DOCK8 protein has GEF function for CDC42 and regulates cell morphology and migration.¹⁹ Although DOCK8-deficient EBV lines expressed S1P receptors similar to wild-type lines (Fig 5, A), the deletion of DOCK8 completely abrogated migration toward S1P. EBV lines carrying homozygous DOCK8 missense mutations (DOCK8-DOCK12) or a deletion of exon 25 (DOCK8-DOCK10) responded to S1P, although less efficiently than wild-type cells (Fig 5, B). Similar to the EBV lines, primary B cells from DOCK8-deficient patients showed normal S1P receptor expression (see Fig E10, A, in this article's Online Repository at www.jacionline.org) but did not migrate as efficiently as control B cells (see Fig E10, B). Therefore DOCK8 seems to function differently in human and mouse B cells because in mice mutations in *DOCK8* did not disturb S1P-induced migration.³²

Downstream of small GTPases, WASp regulates the dynamics of the actin cytoskeleton. WASp-deficient EBV lines expressed normal S1P receptor transcript levels but did not migrate in response to S1P (Fig 5, C and D). However, residual WASp expression in a line carrying a homozygous R86H mutation (Fig E10, C), which prevents the interaction with WIP, allowed normal migration (Fig 5, D). This indicates that even very low amounts of WASp are sufficient to catalyze cytoskeletal reorganization.

Vesicular transport is important for S1P receptor internalization, desensitization, and recycling.³³ LRBA, a member of the BEACH-WD40 protein family, contains WD40 domains that are important for protein-protein interactions, vesicular trafficking, cytoskeletal assembly, and signal transduction.³⁴ Because defects in LRBA impair B-cell activation and result in defective autophagy,²³ we analyzed LRBA-deficient EBV lines carrying a homozygous Ile2657Ser substitution. These cells did not migrate (Fig 5, E) but internalized S1P1 as efficiently as control EBV lines (see Video E6 in this article's Online Repository at www.jacionline.org) without expressing increased levels of CD69⁺ (see Fig E10, D), which might have inhibited S1P1 activity.

Collectively, these data show that 3 genes causing primary immunodeficiencies, *DOCK8*, *LRBA*, and *WAS*, encode proteins that are essential components of the S1P1 signaling pathway.

DISCUSSION

Although B-cell circulation plays an important role in immune responses and immunopathology, S1P receptor expression and function of human B cells was largely unexplored. We found that different from mice,⁷ human B cells do not express S1P3, whereas S1P1, S1P2, and S1P4 are expressed at different levels and ratios by different B-cell subpopulations. Except for PCs, S1P1 was found to promote S1P-dependent migration and egress from bone marrow and from secondary lymphoid tissues for all B-cell subsets. Surprisingly, circulating PCs of FTY720-treated patients with MS did not migrate against S1P. Therefore these PCs might exit from lymphoid tissues by S1P1-independent signals.

In mice activation of S1P2 prevents the response of GC B cells to CXCL12 and CXCL13 by inhibiting CXCR4 and CXCR5 and the exit of cells from GCs.⁹ Here we show that S1P2 is expressed in human GC B cells, as well as in PCs and human B-cell tumor lines. Because S1P2 inhibited S1P-dependent *in vitro* migration

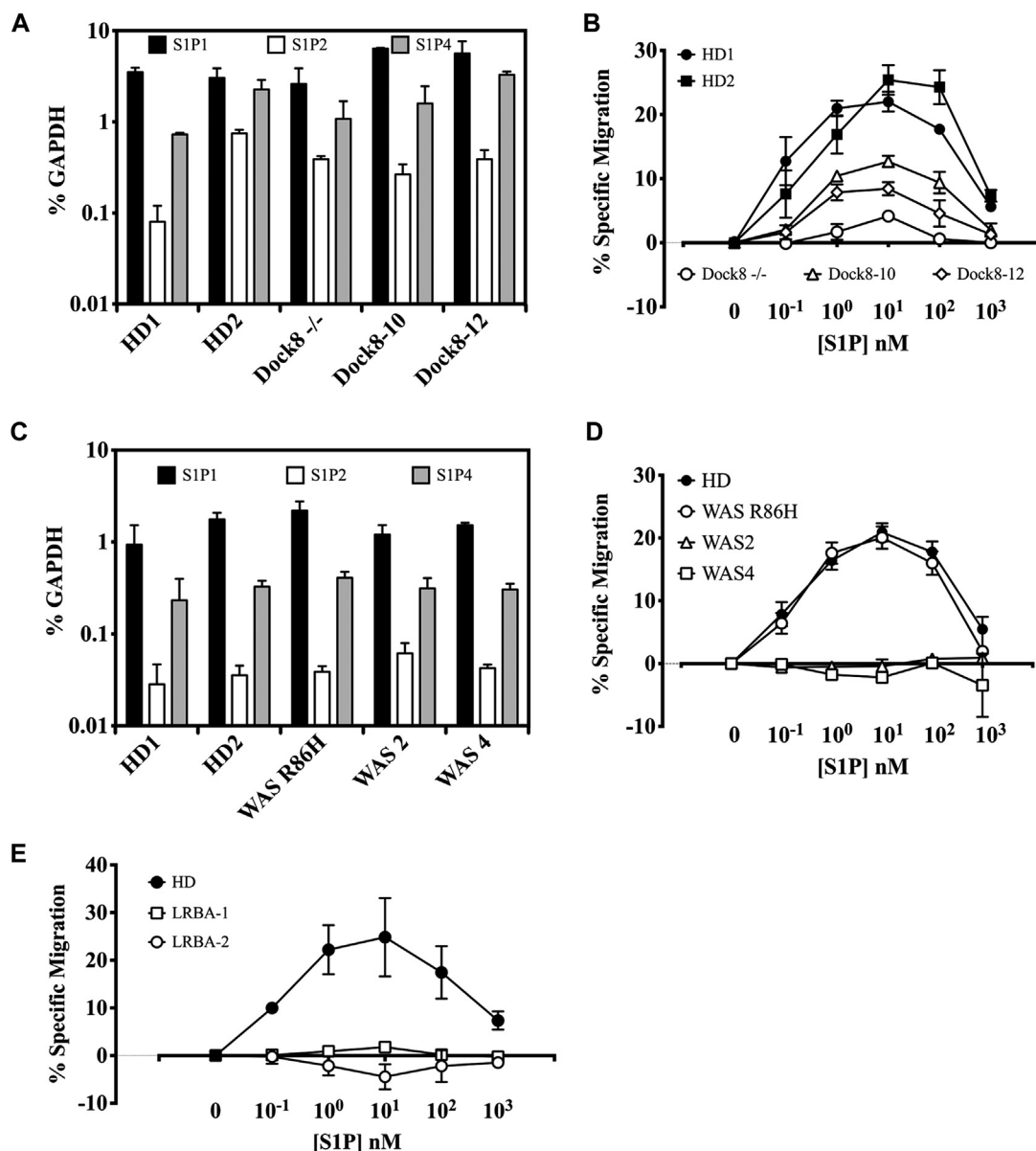


FIG 5. DOCK8, LRBA, and WASp regulate S1P-dependent migration. **A** and **C**, S1P receptor mRNA expression in EBV-immortalized DOCK8-deficient (Fig 5, **A**) and WASp-deficient (Fig 5, **C**) B cells. Values are means $\pm$ SDs of 2 technical replicates. **B**, **D**, and **E**, Migration of DOCK8-deficient (Fig 5, **B**), WASp-deficient (Fig 5, **D**), and LRBA-deficient (Fig 5, **E**) EBV cell lines. Values are means $\pm$ SEMs of at least 3 biological replicates. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase

of S1P1⁺ B-cell lines and of S1P2^{high}S1P1^{lo} GCs and PCs, S1P2 most likely prevents the emigration of S1P2⁺ PCs from tonsils.

In the S1P2 signaling cascade, α_{12} - α_{13} -mediated activation of p115RhoGEF activates RhoGTPase, which in turn antagonizes Akt activation and blocks migration.⁹ RhoGAPs counterbalance RhoGTPases by increasing their intrinsic GTPase activity. Interestingly, the absence of the RhoGAP ARHGAP4, which is highly expressed in hematopoietic cells and was shown to be a negative regulator of cell motility, had an effect on neither the S1P1-mediated migration nor the S1P2-mediated inhibition of migration. In addition, inhibition of the ROCK pathway, which was shown to restore S1P1 migration of S1P2⁺ glioblastoma cells,³¹ had little effect on S1P2-dependent inhibition of B-cell

migration. This suggests that other RhoGTPases are active downstream of S1P2 and that ROCK is not participating in the signaling pathway of S1P2 in human B cells.

Toll-like receptor (TLR) activation in mouse B1 cells strongly impairs S1P-mediated migration.³⁵ In human B cells activation of BCR, CD40, or TLR9 inhibited S1P-dependent migration, downregulated S1P1 transcription, and upregulated the expression of CD69, a direct inhibitor of S1P1 function.²⁷ Nevertheless, resting CD69⁺ primary B cells were still able to migrate, although with lower efficiency, indicating that CD69 expression alone is not sufficient to block S1P1-induced migration. Similar to activated B cells, CLL cells did not migrate effectively against S1P. Inhibition of BCR-dependent cellular activation of CLL cells

by the irreversible BTK inhibitor PCI-32765 leads to the massive exit of leukemic B cells from lymphoid tissues.²⁹ This suggests that BTK inhibition interferes not only with BCR activation and CXCR4- and CXCR5-dependent retention signals but also allows CLL cell egress in response to S1P signals.³⁶ Surprisingly, PCI-32765 treatment blocked the migration of primary resting B cells toward S1P. Therefore BTK activity seems to be required for S1P1 signal transduction, and moreover, PCI-32765-mediated BTK inhibition most likely does not allow S1P-mediated egress of leukemic B cells.

Although S1P4 was expressed by all B-cell subsets, leukemic B cells, and many B-cell lines, the role of this S1P receptor in B cells is still unclear. S1P4 knockout mice did not show major defects in B-cell circulation and development.¹⁰ Overexpression of S1P4-GFP in B-cell lines inhibited S1P1-induced migration, although not to the same extent as observed for S1P2. Internalization studies using TIRFM revealed that triggering of S1P4 results in clustering and internalization, although at a slower rate than observed for S1P1 and S1P2. This suggests that S1P4 acts as a modulator of S1P-mediated chemotaxis.

β -Arrestin 1 and β -arrestin 2 are scaffolds and signal-transducing components of chemokine receptors.³⁷ Overexpression of β -arrestin 2, but not of β -arrestin 1, strongly enhanced migration at low S1P concentrations, suggesting that β -arrestin 2 recruitment is a rate-limiting step in S1P1 signaling. Therefore enhanced β -arrestin 2 expression might promote the dissemination of malignant B cells similar to tumor cells in the murine chronic myelogenous leukemia model.³⁸

In addition to β -arrestin 2, WASp, DOCK8, and LRBA were found to be components of the S1P1 signaling pathway. After binding the GTPase CDC42, WASp is activated³⁹ and initiates actin polymerization, cytoskeletal reorganization, and migration.⁴⁰ Thus WASp-deficient EBV lines did not migrate in response to S1P. Stabilization of WASp through WIP is an essential step in forming actin filaments.⁴¹ Because the R86H WASp mutation prevents interactions with WIP, WASp is highly unstable.⁴² Nevertheless, residual expression of R86H WASp in EBV cells allowed normal migration, showing that interactions between WASp and WIP are not rate limiting for S1P-dependent migration.

DOCK8 acts as a GTP exchange factor⁴³ and adapter in the formation of the B-cell immunologic synapse,³² TLR9-MyD88 signaling,⁴⁴ and dendritic cells as an activator of CDC42,¹⁹ which activates WASp-dependent actin polymerization.³⁹ In contrast to B cells of DOCK8-deficient mice,³² DOCK8-deficient human primary B cells and EBV lines migrated poorly against S1P. Therefore DOCK8 seems to have a nonredundant function in the S1P signaling cascade of human B cells, and impaired S1P receptor signaling might contribute to the clinical manifestations of DOCK8 deficiency.

Mutations in LRBA cause an early onset of primary immunodeficiency accompanied with autoimmunity.²³ The Ile2657Ser LRBA missense mutation resides in one of the β -sheets of the C-terminal WD40 domain, and mutant EBV cells have undetectable LRBA protein levels.²³ Because these cells do not migrate *in vitro* but show normal ligand-induced endocytosis of S1P1, LRBA is not involved in the initial internalization of S1P1 but seems to play a role in downstream signaling.

In conclusion, our data show that β -arrestin 2 regulates the strength of S1P1 signaling. After endocytosis of receptor-carrying vesicles, LRBA is part of the downstream signaling cascade that

initiates S1P-mediated migration in a WASp- and DOCK8-dependent manner. S1P1 signaling is blocked completely by activation of BCR, CD40, TLR9, and S1P2. In addition to these components, S1P4 seems to modulate S1P1 signaling strength. In secondary lymphoid organs these mechanisms limit the mobility of activated B cells and thus control GC reactions during immune responses. The highly increased susceptibility to autoimmunity and lymphoproliferation shared by LRBA and WASp deficiency emphasizes the role of signals, including those induced by S1P receptors, in regulating the organization of the cytoskeleton for normal functioning of the immune system.

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Key messages

- Differential expression of S1P receptors regulates the circulation of human B-cell subsets.
- In human B cells S1P1 receptor 1-induced signals are transmitted through β -arrestin 2, LRBA, DOCK8, and WASp.
- B-cell activation, malignant transformation, and genetic defects in LRBA, DOCK8, and WAS prevent S1P-dependent migration.

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METHODS

Patients

Human peripheral blood was obtained from healthy donors or patients. Spleens and tonsils were obtained from patients undergoing splenectomy or tonsillectomy. All material was used after obtaining informed consent and in accordance with approval 78/2001 of the University Medical Center Ethics Committee.

Cell lines and cell culture

B-lymphoid cell lines were from the German Collection of Micro-organisms and Cell Cultures (Braunschweig, Germany). Dakiki cells were obtained from the ATCC (Teddington, United Kingdom), and EBV cells were generated by infecting PBMCs with wild-type EBV. EBV lines WAS2^{E1} and WAS4^{E2} were kindly provided by S. Scaramuzza and A. Villa. WAS R86H was provided by U. Salzer. DOCK8-deficient EBV lines were either provided by V. Lougaris and A. Plebani (DOCK8^{-/-}) or B. Grimbacher (DOCK8-DOCK10 and DOCK8-DOCK12).^{E3} B. Grimbacher also provided the LRBA line (Ile2657Ser).^{E4} Cells were cultivated at 37°C in a humidified 6.5% CO₂ atmosphere in Iscove modified Dulbecco modified Eagle medium (Invitrogen, Carlsbad, Calif) supplemented with 10% heat-inactivated FCS (Biowest, Nuaille, France), 100 U/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen). Before stimulation with S1P or Transwell migration assays, cells were adapted to S1P-free conditions overnight in migration medium (Iscove modified Dulbecco modified Eagle medium containing 0.5% [wt/vol] fatty acid-free BSA and 10 mmol/L HEPES; Sigma-Aldrich, Deisenhofen, Germany).

Blood and tissue preparation

Human PBMCs were enriched by means of Ficoll density gradient centrifugation (Pan-Biotech, Aidenbach, Germany). For isolation of tonsillar cells, fresh tonsils were squeezed through metal mesh filters followed by singularization through cell strainers (Becton Dickinson, Franklin Lakes, NJ). CD19⁺ B cells were further concentrated by means of negative selection with magnetic cell sorting (MACS) beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Activation of primary B cells

MACS-separated primary human B cells were either seeded into 96-well plates (5×10^4 cells/well) and stimulated overnight with or without anti-IgM (1 µg/mL F[ab']₂; Southern Biotech, Birmingham, Ala), CpG (0.5 µmol/L; APARA Bioscience, Denzlingen, Germany), and CD40 ligand,^{E5} or B cells were seeded into 48-well plates (5×10^5 cells/well) and incubated overnight with dimethyl sulfoxide (DMSO) or PCI-32765 (100 nmol/L in DMSO; Selleckchem, Houston, Tex) with anti-human IgA+IgG+IgM (4 µg/mL; Jackson ImmunoResearch, West Grove, Pa) in migration medium.

Flow cytometry and cell sorting

Cells were stained with anti-CD19–Alexa Fluor 488, anti-CD19–phycoerythrin (PE)–Cy7, or anti-CD19–allophycocyanin-H7; anti-CD27–V450, anti-CD27–fluorescein isothiocyanate (FITC), anti-CD27–PE, anti-CD27–PerCP–Cy5.5 or anti-CD27–PE–Cy7; anti-CD38–FITC, anti-CD38–PE–Cy7, or anti-CD38–PerCP–Cy5.5; anti-CD69–PE; anti-IgD–PE; (BD); anti-IgD–biotin (Southern Biotech, Birmingham, Ala); and anti-IgM–Cy5 (Beckman Coulter, Fullerton, Calif) in PBS (3% FCS and 0.05% NaN₃). Biotinylated antibodies were counterstained with streptavidin–allophycocyanin (BD) or streptavidin–FITC (Dako, Hørsholm, Denmark) in PBS (3% FCS and 0.05% NaN₃). Cells were then acquired with a FACSCanto II flow cytometer (BD) and analyzed with FlowJo software (Tree Star, Ashland, Ore). For sorting, cells were stained in PBS, and tonsillar and blood B-cell subpopulations were purified with a MoFlo XDP cell sorter (Beckman Coulter, Indianapolis, Ind), applying the gating strategy shown in Fig E9.

RNA isolation and qPCR

Total RNA was isolated by means of extraction with Trizol (Invitrogen). Identical amounts of total RNA were applied for cDNA synthesis by using the

reverse transcriptase Superscript III (Invitrogen) and random hexamers (GE Healthcare Europe, Freiburg, Germany). qPCR was performed on a LightCycler 480 (Roche, Basel, Switzerland), with primer and probes sets (Eurogentec, Liege, Belgium) depicted in Table E1. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh* kit, Eurogentec) by using the $2^{-\Delta\text{ct}}$ method.

DNA constructs

Plasmids carrying full-length human β -arrestin 1, β -arrestin 2, S1P1, S1P2, and S1P4 cDNA were obtained from OpenBiosystems (Lafayette, Colo). The DNA fragment containing the S1P1 coding region was cloned into the lentiviral expression vector pNL-CEF-eGFP, which was kindly obtained from Jakob Reiser (LSU, New Orleans). Fusion of S1P1 to enhanced GFP was achieved by inserting a synthetic fragment (Geneart, Regensburg, Germany) bridging the C-terminal part of S1P1 to the N-terminal part of eGFP, skipping the stop codon (Table E1). Coding regions for S1P2 and S1P4 were amplified by means of PCR with primers containing specific enzyme sites (Table E1). Amplified fragments were cloned into the pNL-CEF-S1P1eGFP vector. S1P1 and S1P2 were also ligated into the pTagRFP-N vector (Evrogen, Moscow, Russia). S1PR-RFP was then cloned into the pNL-CEF-S1P1-eGFP vector. The RFP in pNL-CEF-S1P1/2-RFP was further exchanged with BFP (Evrogen) to generate pNL-CEF-S1P1/2-BFP. Coding regions of β -arrestin 1 and β -arrestin 2 were amplified by means of PCR with primer sets containing enzyme sites convenient for ligation into the pTagRFP-N vector (Table E1). Then, β -arrestin 1–RFP and β -arrestin 2–RFP were cloned into the lentiviral backbone. All constructs were verified by sequencing. Enzymes used for cloning were from New England Biolabs (Ipswich, Mass) or from Fermentas (Vilnius, Lithuania).

Transfections and infections

The packaging cell line HEK293T was used to generate lentiviral particles after transfections with the HD Fugene transfection kit (Roche). The packaging vectors pCDNLBH* and pVSV-G, were obtained from J. Reiser and the pNL-S1PR-B/R/G-FP constructs. Supernatants were collected 2 days after transfection and filtered (0.45 µm; Pall Corporation, Port Washington, NY). For stable transduction, 3×10^5 cells were resuspended in 0.5 mL of virus-containing supernatant and spin-infected for 2 hours at 2500 rpm and 30°C.

Preparation of S1P and FTY720P solutions

S1P or FTY720P stock solutions (100 µmol/L) were prepared by adding 0.5% fatty acid-free BSA (Sigma-Aldrich) in H₂O to lyophilized S1P or FTY720P. The suspension was incubated at 37°C for 30 minutes and sonicated for 10 minutes (Amp, 70%; Puls, 0.2 seconds; pause, 0.5 seconds).

Transwell migration assays

Cells ($100 \mu\text{L}$ of $1.5\text{--}2 \times 10^6$ cells/mL) in migration medium were added to the upper chamber of a Transwell (Millipore, Billerica, Mass). The lower chamber contained 500 µL of medium with indicated concentrations of S1P or FTY720P. After 4 hours of migration at 37°C, cells from the upper and lower chambers were collected separately, stained, and recorded by using flow cytometry with a FACSCanto II flow cytometer (BD) and timed acquisition. For rho kinase inhibition experiments, transduced Ramos cells were seeded into 24-well plates incubated overnight with or without rho kinase inhibitor (H1152P) or rho kinase inhibitor IV (H1152P Glycyl, 500 nmol/L; EMD Millipore Corporation, Billerica, Mass) in migration medium. For each measurement, the specific migration was calculated as follows:

$$\left[\frac{((\text{Number of cells in lower chamber})/(\text{Number of cells in lower} + \text{upper chamber}) \times 100) - (\text{Migration at 0 nmol/L attractant}) \right].$$

Immunofluorescence and fluorescence microscopy

Cryosections (10 µm) of human tonsils or spleens were air-dried, fixed in ice-cold acetone, and blocked with 5% goat serum (Vector Laboratories, Burlingame, Calif) in PBS (1 hour at room temperature). A polyclonal rabbit anti-human EDG-1/S1P1 antibody was used for S1P1 detection (H60; Santa

Cruz Biotechnology, Santa Cruz, Calif) together with biotinylated goat anti-IgD (SouthernBiotech, Birmingham, Ala); mouse monoclonal anti-CD3 (Okt3), anti-CD38, anti-Ki67, and anti-CD19–AmCyan (BD); and anti-IgM–Alexa Fluor 594 (Invitrogen). Goat anti-mouse IgG-FITC (SouthernBiotech), goat anti-rabbit IgG Alexa Fluor 568, goat anti-mouse IgG Alexa Fluor 405, Alexa Fluor 405–conjugated streptavidin (Invitrogen), and FITC-conjugated streptavidin (Dako) were used as second-step reagents. Samples were stained in PBS (5% goat serum) for 1 hour at room temperature (or 4°C overnight, first step only) with the first- and second-step reagents washed 3 times with PBS after each step and mounted with Fluoromount-G (SouthernBiotech).

Stable transduced cells or cell lines were placed on collagen-coated cell-culture dishes (Greiner, Frickenhausen, Germany). Internalization of S1P receptor fusion proteins was triggered by addition of S1P or FTY720P to 100 nmol/L into the cell solution on the heated (37°C) fluorescence microscope under a CO₂ atmosphere (6.5%). Images were taken with the inverted microscope Axio Observer (Zeiss, Oberkochen, Germany) by using AxioCam MRm (Zeiss), objective lenses EC Plan-Neofluar 10×/0.3, EC Plan-Neofluar 20×/0.5, EC Plan-Neofluar 40×/0.75, and AxioVision software (version 4.8.2.0). Images were either processed with AxioVision software or Adobe Photoshop CS5 software. Pearson coefficients were determined by using the JACoP plug-in^{E6} of ImageJ software (National Institutes of Health, Bethesda, Md). Live-cell TIRF microscopy was performed with a Nikon Ti-E microscope equipped with an Apochromat TIRF objective lens (oil immersion, magnification = 100×, numerical aperture = 1.49; Nikon, Düsseldorf, Germany). Fluorescence of BFP, GFP, and RFP was excited with 405-nm, 488-nm, and 561-nm lasers, respectively (Coherent, Dieburg, Germany). Images were recorded on an EMCCD camera (Ixon3 DU-897; Andor, Belfast, United Kingdom). Time-lapse image sequences were processed and analyzed with Nikon NIS-elements software. The experiments were performed at 37°C.

Western blotting

Lysates from WASp EBV lines were prepared in ice-cold NP-40 lysis buffer (50 mmol/L Tris [pH 8], 1% NP-40, and 150 mmol/L NaCl) containing

protease inhibitor cocktail (Roche). Lysates were cleared by means of centrifugation before subjecting them to separation on SDS-PAGE, blotting, and incubation with antibodies against WASp (B-9, Santa Cruz Biotechnology) and β -actin (Sigma-Aldrich). Horseradish peroxidase–coupled goat anti-mouse IgG (Sigma-Aldrich) was used to visualize proteins by using enhanced chemiluminescent substrate (Pierce ECL, Thermo Scientific, Uppsala, Sweden).

Statistical analysis

Between groups of healthy donors and patients or transduced BJAB cells, a 1-way ANOVA Kruskal-Wallis test was performed, and between groups of differently stimulated B cells, a 1-way ANOVA Bonferroni multiple-comparison test was performed with Prism software (GraphPad Software, La Jolla, Calif) to determine statistical significance. A *P* value of less than .05 was considered significant.

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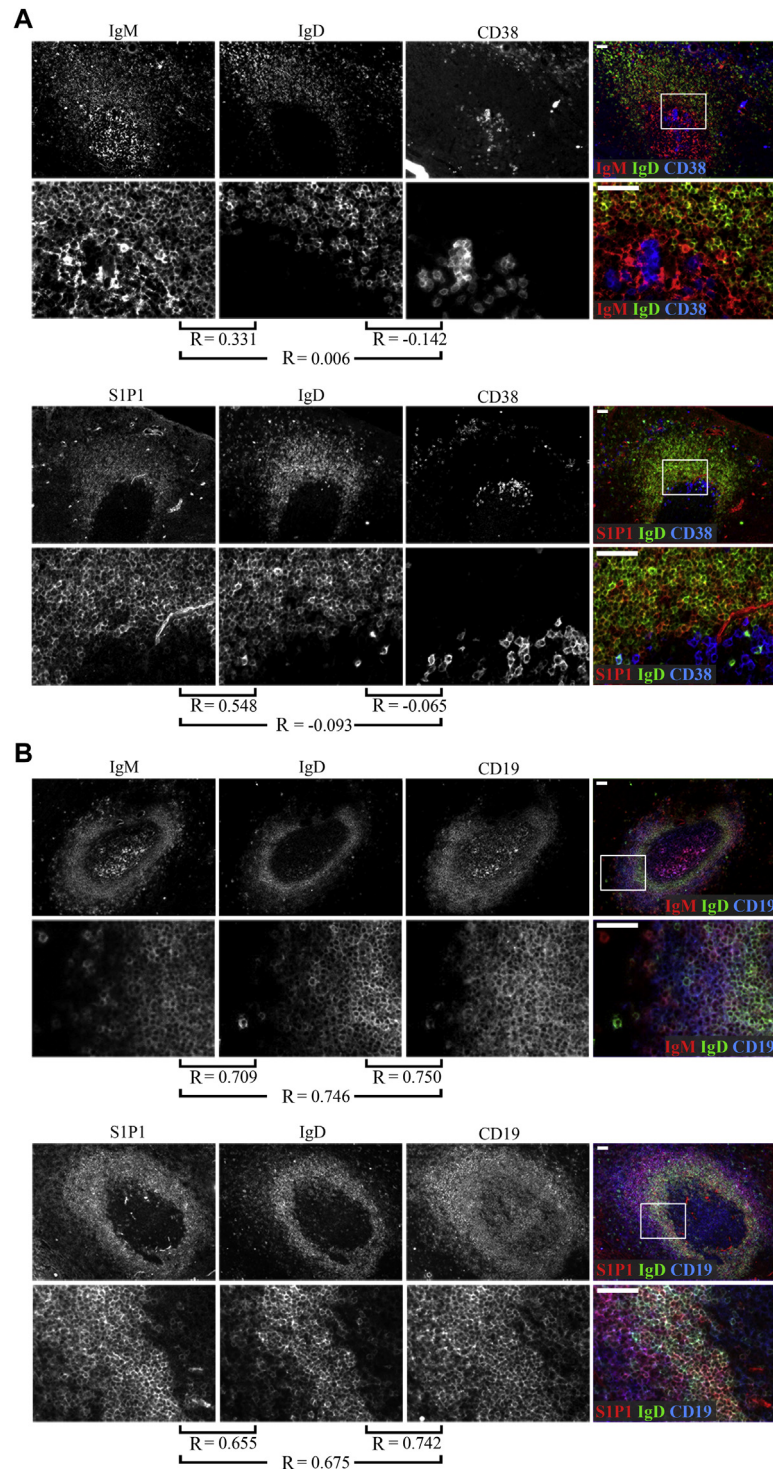


FIG E1. S1P receptors in human B cells. Immunofluorescence microscopy of tonsillar (**A**) and spleen (**B**) cryosections stained with anti-IgD (green), anti-CD19 or anti-CD38 (blue), and anti-IgM or S1P1 (red). Scale bars = 50 μ m. *R*, Pearson coefficient.

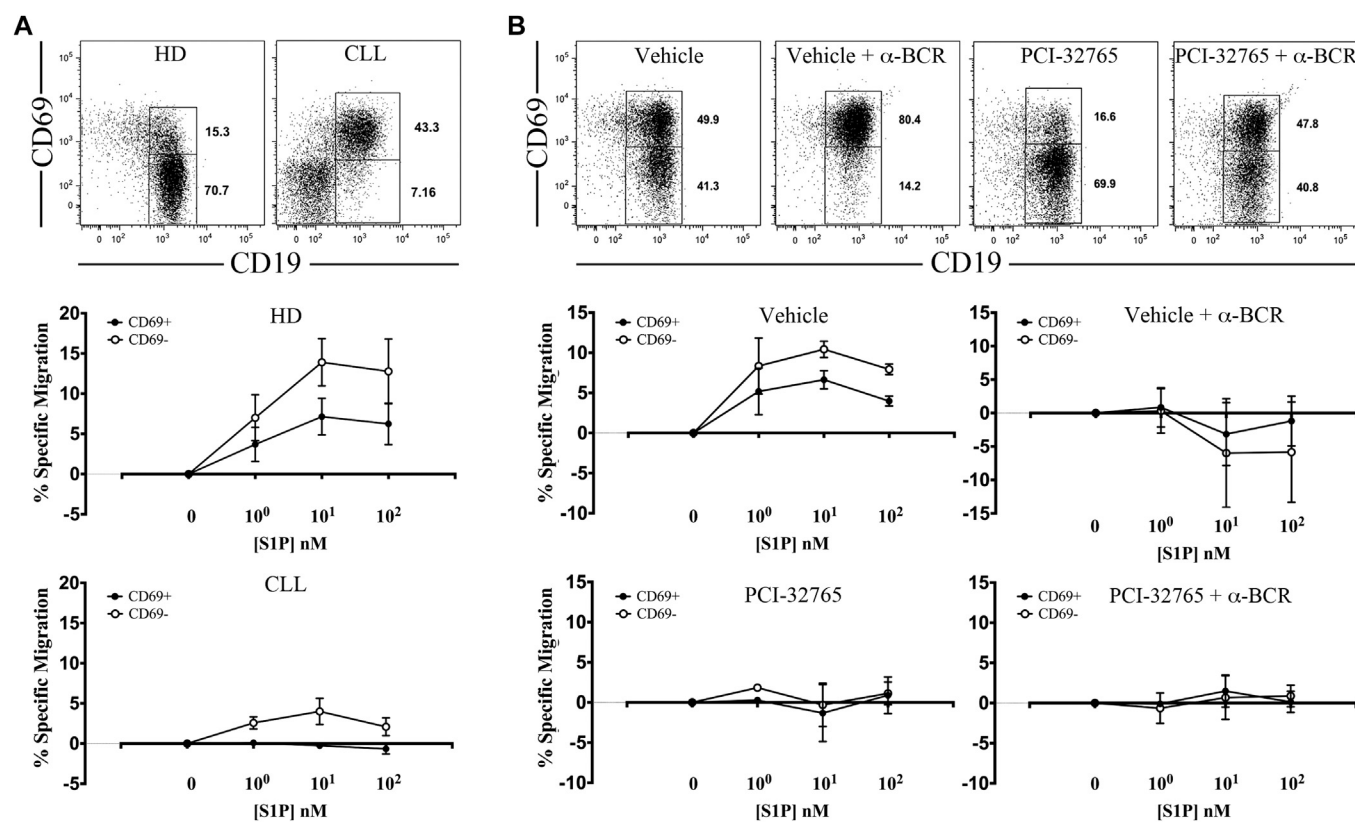


FIG E2. CD69 expression and inhibition of BTK activity impair S1P-induced B-cell migration. **A**, Migration of primary B cells and CLL cells gated for CD69 expression. Values are means $\pm$ SEMs of 3 (healthy donor [HD]) and 8 (CLL) biological replicates from 5 different patients. **B**, Migration of primary CD69⁺ and CD69⁻ B cells 14 hours after BCR activation $\pm$ 100 nmol/L PCI-32765 or DMSO (1:10,000; vehicle). Values are means $\pm$ SEMs of 3 different biological replicates.

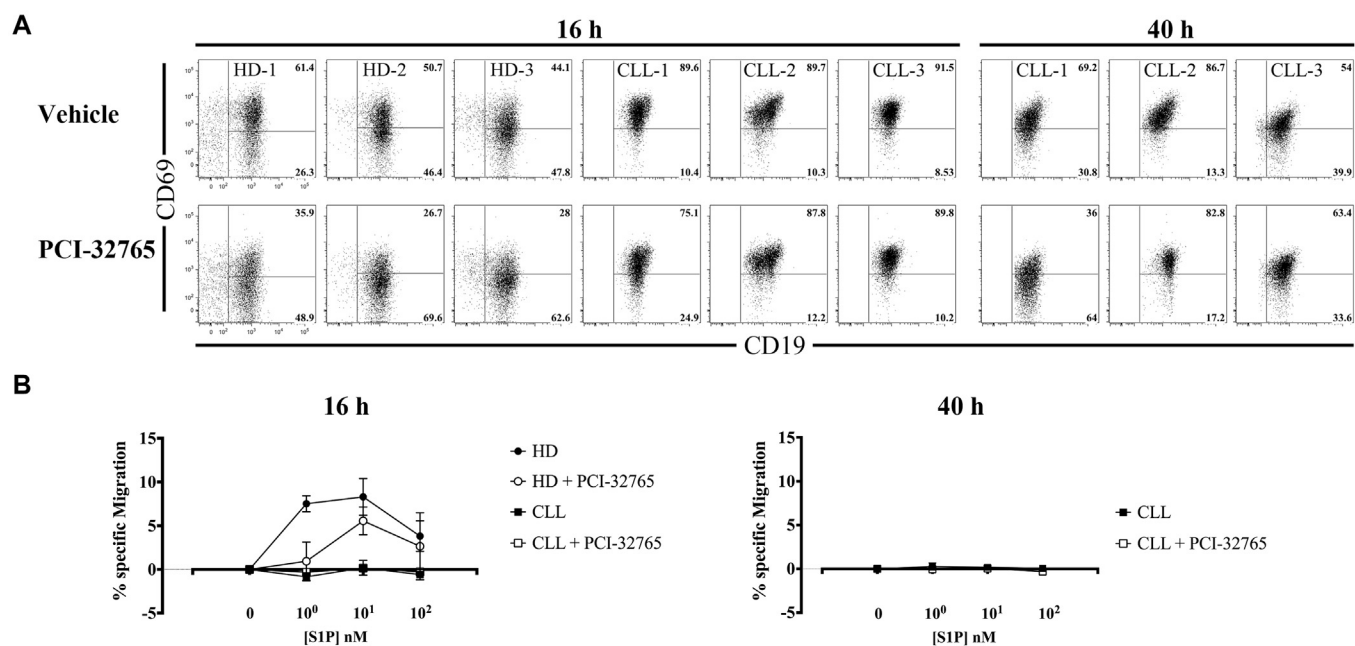


FIG E3. Inhibition of BTK does not restore S1P-induced migration in CLL cells. **A**, Fluorescence-activated cell sorting analysis of CD69 expression of healthy donor (HD) and CLL cells treated with PCI-32765 for 16 and 40 hours. **B**, Migration of primary B cells and CLL cells treated with PCI-32765. Values are means $\pm$ SEMs of 3 biological replicates: PCI-32765 (100 nmol/L) or DMSO (1:10,000; vehicle).

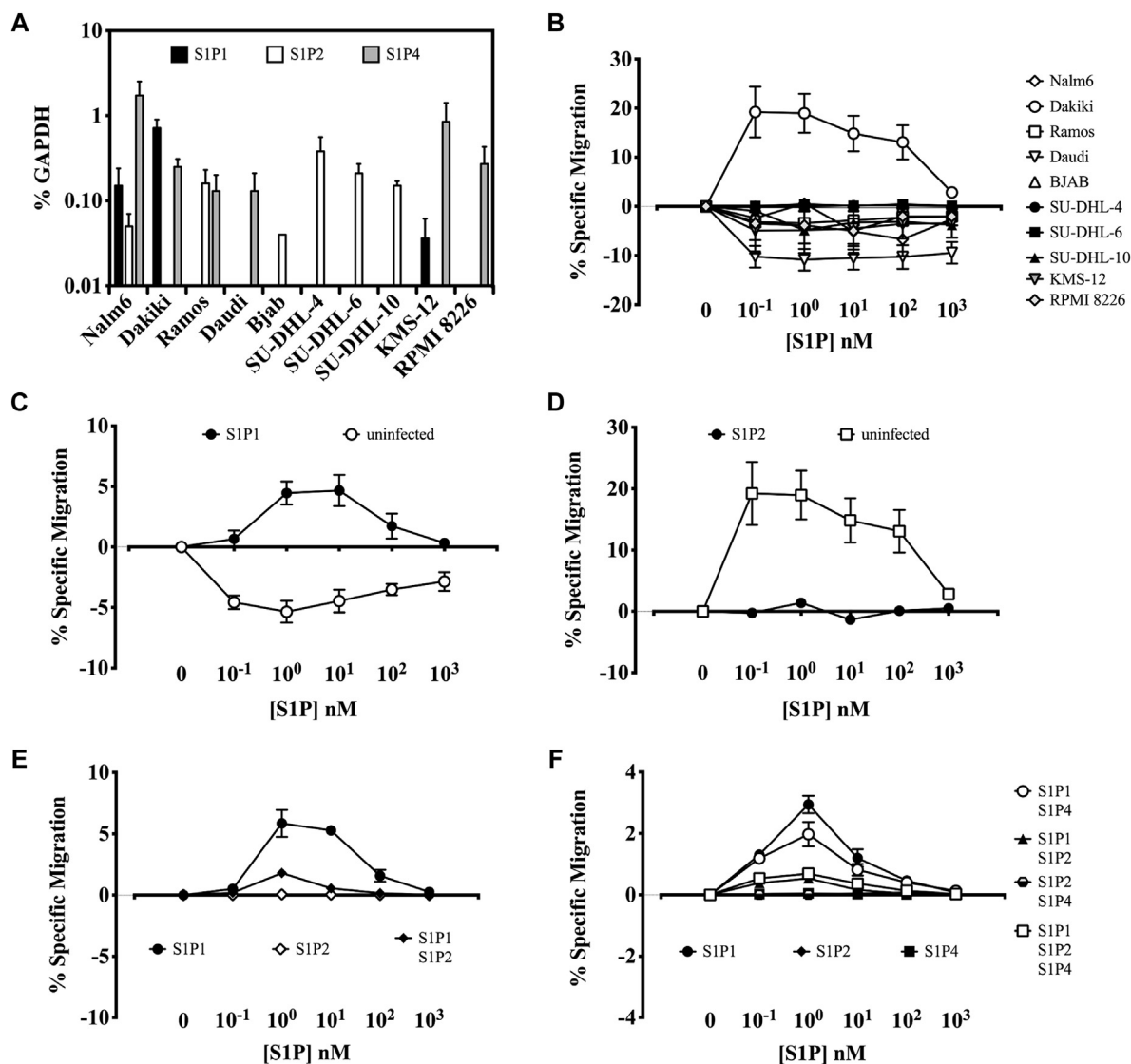


FIG E4. S1P receptor expression and migration of human B-cell tumor lines. **A**, S1P receptor mRNA expression in B-cell lines. Values are means $\pm$ SDs of 2 different technical replicates. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase. **B-F**, Migration of B-cell lines (Fig E4, **B**), S1P1-transduced Ramos cells (Fig E4, **C**), S1P2-transduced Dakiki cells (Fig E4, **D**), S1P1- and S1P2-transduced BJAB cells (Fig E4, **E**), and S1P1-, S1P2-, and S1P4-transduced BJAB cells (Fig E4, **F**). Values are means $\pm$ SEMs of at least 3 (Fig E4, **B** and **D-F**) and 5 (Fig E4, **C**) experimental replicates.

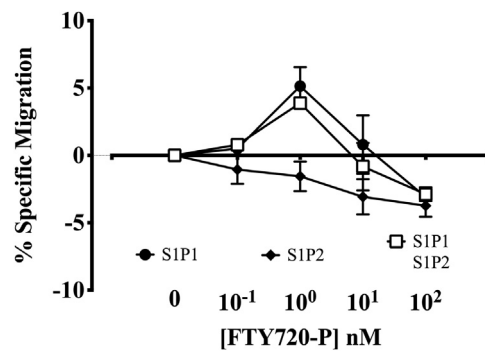


FIG E5. S1P2 does not inhibit FTY720P-induced S1P1-dependent migration. Migration of S1P1- and S1P2-transduced Ramos cells against FTY720P was analyzed in Transwell assays. Values are means $\pm$ SEMs of 3 biological replicates.

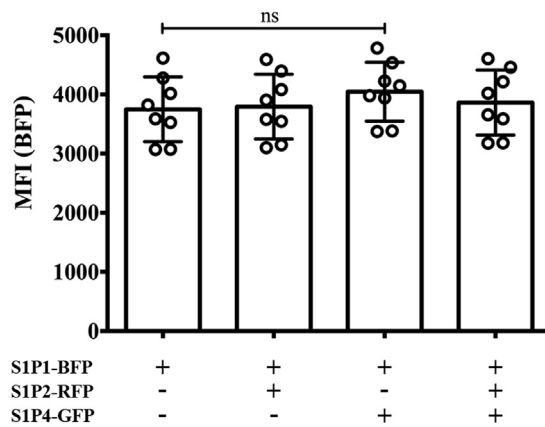


FIG E6. S1P1-BFP expression is not changed in S1P2-RFP- and S1P4-GFP-cotransduced BJAB cells. Mean fluorescence intensity (*MFI*) of S1P1-BFP expression in cotransduced BJAB cells measured by means of flow cytometry is shown. Values are means $\pm$ SEMs. Each *dot* represents 1 measurement. *ns*, Not significant.

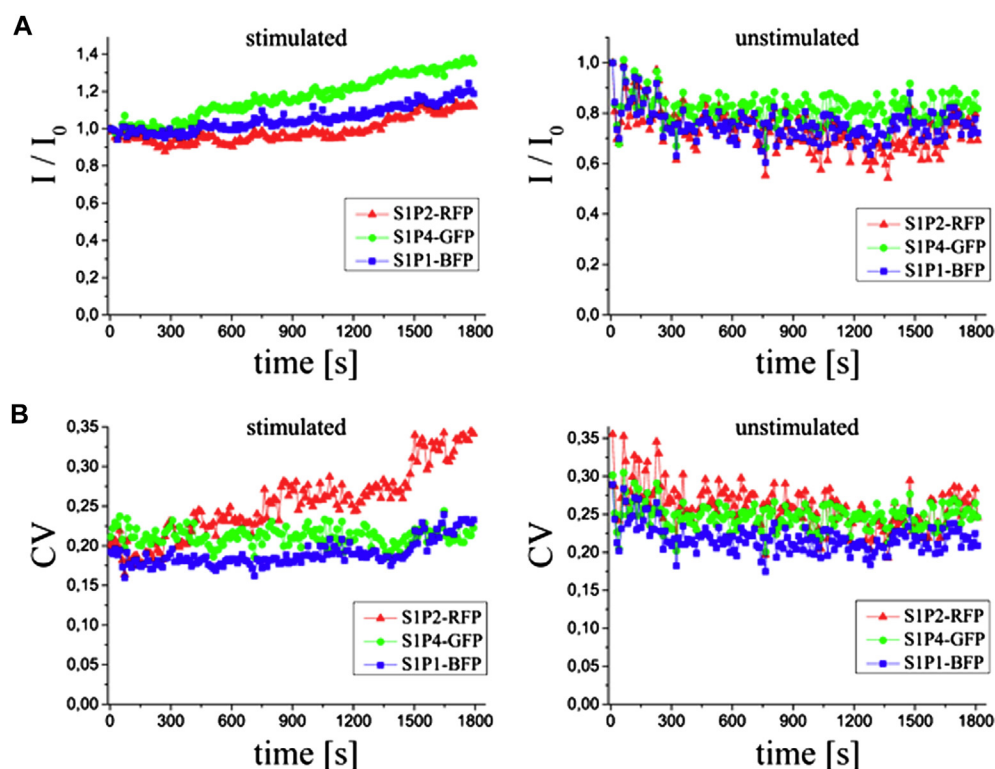


FIG E7. Mean signal intensities and coefficient of variation (CV) of TIRF images. We quantified time-lapse images of TIRF microscopy by calculating the mean signal intensities and SDs per pixel for each channel for unstimulated and S1P-stimulated cells. **A**, Stimulated cells show a constant increase for S1P1-BFP, S1P4-GFP, and S1P2-RFP signals. This is caused by an increase of the contact area between the cell and the microscope slide and, in addition, by increased vesicle formation and the recruitment of S1P receptor fusion proteins to the contact area that increases to start migration. **B**, Plots of the CV (SD/mean intensity) for each channel. Because activation with S1P induces clustering and vesicle formation, this process is accompanied by an increased CV. The CV of S1P2-RFP shows the most rapid change, followed by S1P1-BFP and S1P4-GFP. These changes are not observed in unstimulated cells.

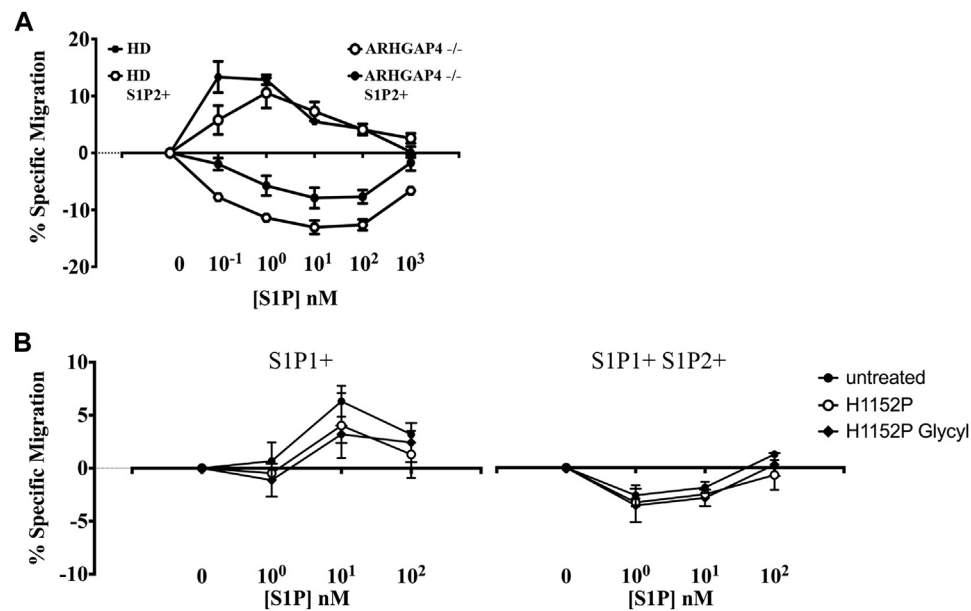


FIG E8. Regulation of S1P-dependent migration by ARHGAP4 and ROCK inhibition. **A**, Migration of S1P2-transduced ARHGAP4-deficient EBV cells toward S1P. **B**, Migration of S1P1- and S1P2-transduced Ramos cells $\pm$ 500 nmol/L H1152P (*Glycyl*) or DMSO toward S1P. Values are means $\pm$ SEMs of 3 (Fig E8, A) and 2 (Fig E8, B) biological replicates.

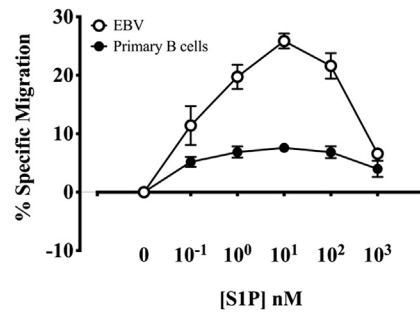


FIG E9. Comparison of primary B-cell and EBV cell migration. Migration of 10 randomly selected primary B cells and 10 randomly chosen EBV-immortalized B cells toward S1P. Values are means $\pm$ SEMs of 10 different replicates.

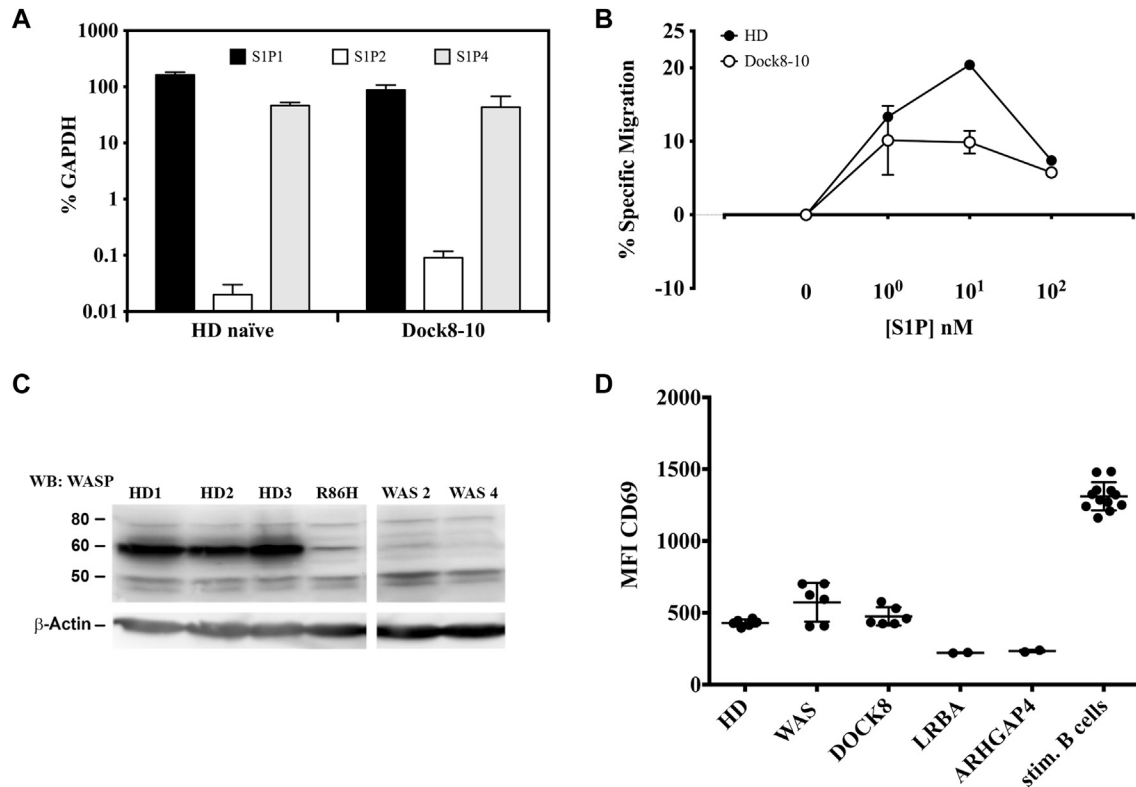


FIG E10. S1P receptor expression and migration of DOCK8-defective B cells and WASp and CD69 expression. **A**, S1P receptor mRNA expression in primary DOCK8-deficient B cells was analyzed by using qPCR. Values are means $\pm$ SDs of 2 different biological replicates. **B**, Migration of primary DOCK8-deficient B cells analyzed in Transwell assays. Values are means $\pm$ SEMs of 3 biological replicates. **C**, Western blot analysis of WASp expression in EBV lines carrying WAS deletions or the R86H missense mutation impairing WIP interactions. **D**, Mean fluorescence intensity (MFI) of CD69 expression as analyzed by using flow cytometry in EBV lines and activated B cells. Different acquisitions are represented by individual symbols; the means are indicated by horizontal lines. HD, Healthy donors.

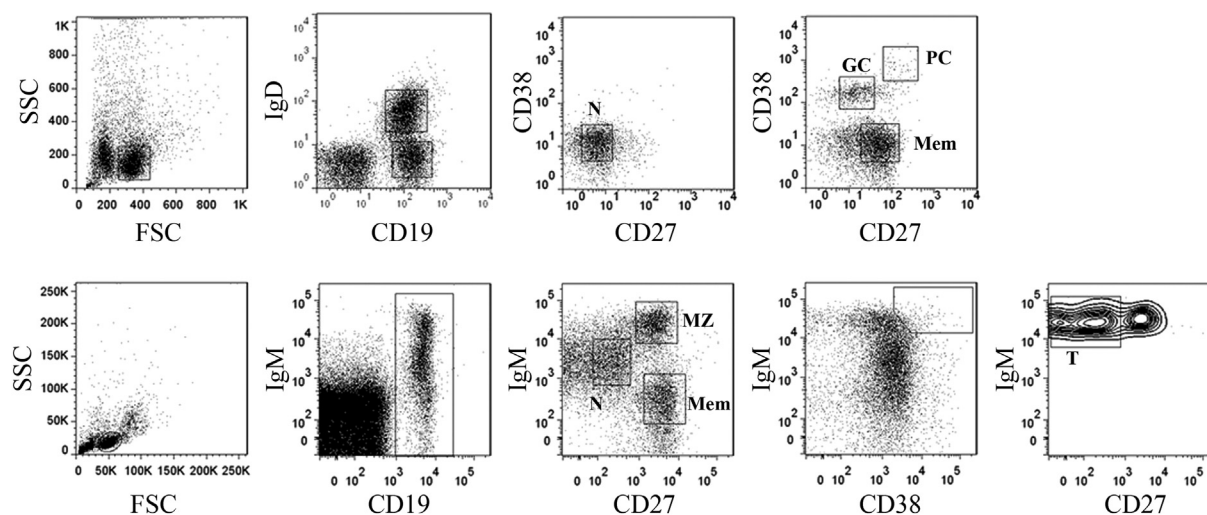


FIG E11. Sorting strategy of isolated B cells. Human primary tonsillar (*top*) and blood (*bottom*) B-cell subsets were defined according to forward scatter (FSC) and side scatter (SSC). Tonsillar cells within the lymphocyte gate were separated according to CD19 IgD staining into CD19⁺IgD⁻ and CD19⁺IgD⁺ fractions. CD19⁺IgD⁻ cells were further separated into fractions of CD38^{hi}CD27⁻ GCs and CD27⁺CD38^{-/lo} memory B cells (*mem*). Naive CD19⁺IgD⁺ B lymphocytes were collected as CD27⁻CD38^{-/lo} cells. Blood B-cell subsets were enriched first from cell suspensions by depleting CD19⁻ cells with a MACS. Then cells were stained with anti-CD19, anti-IgM, and anti-CD27 antibodies. The fractions of naive (CD19⁺IgM^{int}CD27⁻), MZ (CD19⁺IgM⁺CD27⁺), and memory (CD19⁺IgM⁺CD27⁺) B cells were collected and used for the isolation of total RNA. Transitional B cells were identified by gating first on CD19⁺IgM^{hi}CD38^{hi} cells followed by gating on IgM^{hi}CD27⁻ cells.

TABLE E1. qPCR primer and probe sequences

Primer	Sequence
S1P1 forward	5'-GCTCTCCGAACGCAACTTC-3'
S1P1 reverse	5'-CGATGAGTGATCCAGGCTTT-3'
S1P1 probe	5'-FAM-TGAGCGAGGCTGCGGTTTCC-TAMRA-3'
S1P2 forward	5'-GGACGCAGACGCCAAG-3'
S1P2 reverse	5'-ATGGTGGGGCTCAGAGG-3'
S1P2 probe	5'-FAM-CCTAGCCAGTTCTGAAAGCCCCA-TAMRA-3'
S1P3 forward	5'-CGCCACCCGCTAGGAT-3'
S1P3 reverse	5'-CTGAGGTTCCGGCTTCATT-3'
S1P3 probe	5'-FAM-AGCCGACGGAGGAGCCCTTT-TAMRA-3'
S1P4 forward	5'-TCTGCCCCCTCTACTCCAAGC-3'
S1P4 reverse	5'-CCATAGAGGCCCATGATGGTG-3'
S1P4 probe	5'-FAM-CCTCTTCTGCCTGGTGATCTTCGCCG-TAMRA-3'
S1P5 forward 1	5'-GCCCAGGAAGCTCAGTTCAC-3'
S1P5 forward 2	5'-TTCAAGGCAGCGCGACTG-3'
S1P5 reverse	5'-GTAGTTGTAATGCAGGACGATGAC-3'
S1P5 probe	5'-FAM-CCGCAGCAGCCCCGACTCCAT-TAMRA-3'
S1P2-AgeI	5'-TCATCTACCGGTGCCATGGGCAGCTTGTACTCGG-3'
S1P2-BamHI	5'-CGGTGGATCCCTGACCACCGTGTGGCCCTC-3'
S1P4-AgeI	5'-TCATCTACCGGTGCCATGAACGCCACGGGGA-3'
S1P4-BglII	5'-GGAAGATCTCGGATGCTCCGCACGCTG-3'
S1P1-GFP	GAGGAATTCAGCCGCAGCAAATCGGACAATTCCTC
linker	CCACCCCCAGAAAGACGAAGGGGACAACCCAGA GACCATTATGTCTTCTGGAAACGTCAACTCTTCTT CCAGGGATCCAC

VIDEO E1. FTY720P-induced internalization of S1P1 but not S1P2. S1P1-GFP- and S1P2-BFP-transduced Ramos cells were transferred to collagen-coated glass-bottom dishes, adapted to S1P-free medium for 16 hours, and stimulated by the addition of FTY720P to 100 nmol/L. Time-lapse epifluorescence microscopy shows internalization of S1P1-GFP, whereas S1P2-BFP remains on the plasma membrane. Images were taken for 60 minutes at 1 per minute. The video is displayed at a display rate of 12 frames per second. The *white arrow* points to a cell internalizing S1P1 (*green*). *Scale bar* = 20 μm .

VIDEO E2. Membrane dynamics of S1P1, S1P2, and S1P4 in unstimulated cells. S1P1-BFP-, S1P2-RFP-, and S1P4-GFP-transduced BJAB cells were transferred to collagen-coated glass-bottom dishes and adapted to S1P-free medium for 16 hours. Time-lapse TIRFM showing membrane dynamics under unstimulated conditions. Images were taken for 30 minutes at 0.09 frames per second. The video displays 12 frames per second. *Scale bar* = 5 μm .

VIDEO E3. S1P-induced internalization of S1P1, S1P2, and S1P4. S1P1-BFP-, S1P2-RFP-, and S1P4-GFP-transduced BJAB cells were transferred to collagen-coated glass-bottom dishes and adapted to S1P-free medium for 16 hours. Time-lapse TIRFM shows membrane dynamics in S1P-stimulated cells (100 nmol/L S1P). Stimulation induces clustering and internalization of S1P1, S1P2, and S1P4 fusion proteins. The *yellow arrows* point to membrane areas where S1P1-BFP, S1P2-RFP, and S1P4-GFP colocalize. Images were taken for 30 minutes at 0.08 frames per second. The video has a display rate of 12 frames per second. *Scale bar* = 5 μm .

VIDEO E4. S1P-induced internalization of S1P1, S1P2, and S1P4 and colocalization with β -arrestin 2. S1P1-GFP- and S1P2-BFP-transduced or S1P1-BFP- and S1P4-GFP-transduced and β -arrestin 2-RFP-transduced Ramos cells were transferred to collagen-coated glass-bottom dishes, adapted to S1P-free medium for 16 hours, and stimulated by the addition

of S1P to 100 nmol/L. Time-lapse epifluorescence microscopy showing internalization and colocalization of fusion proteins into the same vesicles. Images were taken for 60 minutes at 1 per minute. The video is shown with a display rate of 12 frames per second. *White arrows* in the left video point to examples at which internalization of S1P1 (*green*) and S1P2 (*blue*) and colocalization with β -arrestin 2 (*red*) can be observed. The *white arrow* in the right video points to a characteristic example showing internalization and colocalization of S1P1 (*blue*) and S1P4 (*green*) with β -arrestin 2 (*red*). The *green arrow* shows an example at which internalization of S1P4 (*green*) and colocalization with β -arrestin 2 (*red*) is observed. *Scale bar* = 20 μm .

VIDEO E5. S1P-induced internalization of S1P1, S1P2, and S1P4 and colocalization with β -arrestin 1. S1P1-GFP- and S1P2-BFP-transduced or S1P1-BFP- and S1P4-GFP-transduced and β -arrestin 1-RFP-transduced Ramos cells were transferred to collagen-coated glass-bottom dishes adapted to S1P-free medium for 16 hours and stimulated by the addition of S1P to 100 nmol/L. Time-lapse epifluorescence microscopy showing internalization and colocalization of fusion proteins into the same vesicles. Images were taken for 60 minutes at 1 per minute. The display rate is 12 frames per second. The *white arrow* in the left video points to an example in which internalization of S1P1 (*green*) and S1P2 (*blue*) and colocalization with β -arrestin 1 (*red*) can be observed. The *white arrow* in the right video points to a cell internalizing and colocalizing S1P1 (*blue*) and S1P4 (*green*) with β -arrestin 1 (*red*). The *green arrow* points to a typical example at which internalization of S1P4 (*green*) and colocalization with β -arrestin 1 (*red*) can be observed. *Scale bar* = 20 μm .

VIDEO E6. S1P-induced internalization of S1P1 in wild-type and LRBA-deficient EBV lines. S1P1-GFP-transduced wild-type or LRBA-deficient cells were transferred to collagen-coated glass-bottom dishes, adapted to S1P-free medium for 16 hours, and stimulated by the addition of S1P to 100 nmol/L. Time-lapse epifluorescence microscopy showing internalization of S1P1-GFP fusion proteins. Images were taken for 60 minutes at 1 per minute. The display rate is 12 frames per second. *Scale bars* = 50 μm .